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Short communication

Assessing the antibiofouling potential of a fullerene-coated surface

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ABSTRACT

Antibacterial fullerene-based particles, termed nC_{60}, were coated onto a polystyrene surface to evaluate their ability to prevent biofilm formation by *Pseudomonas mendocina*. Biofilm growth on this surface was assessed using ethidium bromide staining and SEM, and cell viability was determined using live/dead fluorescent cell staining. Unexpectedly, surfaces coated with nC_{60} developed a biofilm earlier than the uncoated control, and a higher percentage of live bacteria. This shows that some antimicrobial nanomaterials may lose their efficacy when applied as coatings. The nC_{60} coating appeared to encourage rather than discourage biofilm formation. Furthermore, the bacteria that adhered to the surface were not killed, implying that while nC_{60} would not perform well in this application, the electronic properties of fullerenes and their apparent ability to encourage biofilm formation should be investigated for potential microbial fuel cell applications.

1. Introduction

Biofilms are a fascinating example of unicellular organisms living a multicellular lifestyle. Bacteria in biofilms undergo phenotypic changes to adapt from a planktonic or free-floating lifestyle to a sessile or attached one; genetic regulation controls their behavior when in the biofilm to shift focus from individual survival to group survival. Biofouling, or the undesirable accumulation of organic growth on surfaces that come in contact with water, is often the result of biofilm formation. This phenomenon adversely affects industrial applications, the food industry, and medical applications, and it has been extensively studied and reviewed (Stoodley et al., 2002; Hall-Stoodley et al., 2004). Biofouling annually costs billions of dollars incurred in the removal of biofilms, replacement of corroded and damaged parts, and loss of productivity.

Biofilm formation is difficult to prevent and even more difficult to remove. The biofilm matrix conserves degraded bacteria and nutrients, and it affords superior resistance to antimicrobial agents, dehydration, UV, and other environmental stressors. Current antibiofouling technologies do not last, nor are they effective against pre-existing biofilms; the best antifoulant is an inhibitor of biofilm formation. Different antifouling strategies exist for different applications. To prevent fouling of catheters and implants in medical applications, the products are either impregnated with antimicrobial agents or have these agents covalently bonded to the surface (Danese, 2002). However, once bacteria have adhered to the surface and died, they act as a protective layer on which other bacteria can attach. In the past, the only effective method to prevent macroscopic growth on seafaring vessels was to mix tributyl tin into the paint on the ships' hulls; however, due to the extreme ecotoxicity of tributyl tin, this use has since been banned (Champ, 2000). As more is learned regarding how bacteria form biofilms, there is also the prospect of interfering with the different cellular processes needed for biofilm formation (Lejeune, 2003). Some marine organisms prevent their bodies from being covered with biofilm by secreting various compounds, such as terpenoids, steroids, and saponins (Fusetani, 2004). These and other types of natural antibiofoulants make promising candidates, but none have proven industrially useful.

The current nanotechnology boom introduces a suite of new materials with antimicrobial properties that could be used as antifouling agents. The fullerene, C_{60}, has antibacterial properties in aqueous suspension, in a fullerene water suspension (FWS) termed nC_{60} (Lyon et al., 2005, 2006, 2008). A fullerene water suspension can be formed either through stirring C_{60} powder in water for extended periods of time or by using an intermediary solvent such as tetrahydrofuran or toluene (Heymann, 1994; Cheng et al., 2004; Andrievsky et al., 1995; Yamakoshi et al., 1994; Deguchi et al., 2001; Fortner et al., 2005). nC_{60} is a potentially attractive antibacterial

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agent due to its potency, broad spectrum of activity, and ability to kill bacteria under light, dark, aerobic, or anaerobic conditions (Lyon et al., 2005, 2006, 2008). nC_{60} has been shown to be effective against the biofilm-formers *Pseudomonas putida*, with a minimal inhibitory concentration (MIC) of 0.25–0.5 mg l^{-1}, and *Pseudomonas aeruginosa*, with an MIC of 0.05–0.066 mg l^{-1} (Fang et al., 2007; Lyon et al., 2008).While nC_{60} might not be the best suited for medical applications, due to its potential deleterious interactions with mammalian cells (Sayes et al., 2004), it could be used in industrial water treatment systems, filtration membranes, or antibiofouling applications. In this paper, we explore the efficacy of nC_{60} as an antibiofouling agent. Polystyrene cell culture plates coated with nC_{60} were exposed to biofilm-forming bacteria, and the growth of biofilm was observed using a combination of staining and microscopy techniques.

2. Materials and methods

2.1. Coating cell culture plates with nC_{60}

The nC_{60} was manufactured as described previously (Fortner et al., 2005). Briefly, 25 mg C_{60} (99.5% pure, MER Corp., Tucson, AZ, USA) were dissolved in 1 l tetrahydrofuran (certified spectra-analyzed, Fisher Scientific, Houston, TX, USA) and then filtered through a 0.22-μm nylon Osmonics filter (Fisher Scientific). Water was added to an equal volume of vigorously stirred C_{60} in tetrahydrofuran at a rate of 750 ml min^{-1}. Tetrahydrofuran in excess was evaporated using a Buchi Rotavapor (Buchi Labortechnik AG, Flawil, Switzerland) to a final volume of 100 ml. This final suspension of nC_{60} was stored overnight before being filtered through a 0.22-μm nylon filter.

The nC_{60} was airbrushed into one-half of the wells of a 24-well Costar® tissue culture plate (Corning Inc., Corning, NY), with each well receiving 1 ml of a 10 mg l^{-1} suspension. The uncoated wells served as controls. During the airbrushing, the plate floated in a bath of boiling water in order to speed the evaporation process and provide an even coat of nC_{60} particles. Once the plates were sprayed, they were allowed to dry overnight. The plates were exposed to UV light in a laminar flow hood for 3 h to sterilize them prior to inoculation with media and bacteria.

2.2. Biofilm growth conditions

Pseudomonas mendocina KR1, a known biofilm-forming bacterium (Jayasekara et al., 1999), was maintained on LB plates. To grow biofilm on the culture plates, 0.5 ml of minimal Davis medium (MD) (Lyon et al., 2005) were transferred into each of the 24 wells of the nC_{60}-coated cell culture plate. For optimal biofilm formation, the *P. mendocina* cells had to be stressed prior to inoculation. A tube of 50% LB medium and 50% water was inoculated with *P. mendocina* from an LB plate. This culture was incubated in a shaking incubator at 37 °C overnight. The culture was then left still at room temperature for at least 36 h prior to use. To monitor biofilm growth, 20 μl of the stressed *P. mendocina* culture were added to the MD media in the culture plates at different time points (0, 4, 8, 12, 16, and 20 h). Each of the six columns of the plate was at a different time points. The plate was set up with one of the four rows being a positive control with no nC_{60}, a negative control with no bacteria and no nC_{60}, a negative control with no bacteria, and an experimental set with bacteria and nC_{60}. The plate was incubated in a 37 °C incubator without shaking. At the end of the growth period, the cells and media were removed by pipetting, and the wells of the plate were rinsed three times with approximately 2 ml distilled water, which was removed by pipetting each time. The plates were stained and viewed immediately as detailed below.

2.3. Biofilm growth detection

Three different methods were used to assess biofilm formation: staining with ethidium bromide, scanning electron microscopy (SEM), and fluorescent microscopy. Instead of crystal violet, ethidium bromide was selected as a stain that allowed quick biofilm detection without also staining the nC_{60} in the background (Djordjevic et al., 2002). Ethidium bromide (0.1%) was used to stain each well for 15 min, after which the stain was removed and the wells rinsed three more times. The plate was then placed in a UV hood and photographed. The brightness of each well was indicative of the amount of bacterial growth. Growth on the bottom of the wells was also analyzed using a FEI Quanta environmental scanning electron microscope (FEI, Hillsboro, OR). The bottoms of the plastic wells were removed by using a heated metal coring device. The resulting plastic disks were then mounted onto SEM stubs using carbon tape and coated with 30 nm of gold prior to viewing in the SEM.

In a complementary assay to determine the viability of the bacteria that formed the biofilm, biofilms were grown on the plates for 0.5, 2, 4, 6, and 12 h, and then stained with live/dead fluorescent dyes that differentiated actively respiring bacteria. The dyes used were 4′,6-diamidino-2-phenylindole (DAPI) to detect all cells by staining nucleic acids and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which is only reduced to a fluorescent compound by actively respiring bacteria (Yu et al., 1995; Rodriguez et al., 1992). The staining procedure was modified from that used by Huang et al. (Huang et al., 1995). Working in a darkened room, the wells were gently rinsed in distilled water three times to remove unattached bacteria. The wells were stained with 500 μl of a 0.05% CTC solution for 1 h at 25 °C and then rinsed gently with distilled water. The wells were immediately stained with 500 μl water containing 1 μl of a 1 g l^{-1} DAPI stock solution for 5 min, and then rinsed again. The wells were cored with a heated corer, and the resulting plastic disks were mounted onto a thin glass slide using nail polish. The samples were examined within 2 h using an Axioskop confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY), taking pictures of five fields of view per sample at 400× magnification.

2.4. Statistical analysis

All experiments were performed at least in triplicate. The pictures shown in Figs. 1 and 2 are based on one representative replicate. Student's *t*-test was used to identify statistically significant differences at a 95% confidence level.

3. Results and discussion

nC_{60} behaves as a broad-spectrum antimicrobial agent and has already been shown to prevent the growth of bacteria in suspension (Lyon et al., 2005, 2006, 2008; Fang et al., 2007). This experiment was aimed at taking advantage of the antimicrobial properties of nC_{60} to inhibit biofilm formation. Two different methods, staining with ethidium bromide and SEM, were used to assess biofilm formation. Preliminary tests were performed to determine the time at which biofilm growth could first be distinguished. These tests used ethidium bromide, which would stain nucleic acids in the cells of the biofilm but not stain the nC_{60}.

Fig. 1 shows a plate inoculated with *P. mendocina* for 24 h, stained with ethidium bromide, and visualized under UV to determine which wells had biofilm formation. The controls with no bacteria showed no fluorescence and thus no biofilm. The control with bacteria showed biofilm growth after 20 h, and the wells coated with nC_{60} showed growth even sooner at 16 h. Based on the formation of biofilm at an earlier time point in wells containing nC_{60} as compared to the controls, it appears that (surprisingly) nC_{60} encouraged rather than hindered biofilm formation.

The wells from this experiment were cored and mounted for SEM analysis (Fig. 2). The SEM micrographs chronicle the formation of the biofilm on the nC_{60} layer. The time 0 micrographs show the nC_{60} layer, with the individual nC_{60} crystals being similar in shape and size to those pictured previously by TEM (Fortner et al., 2005). At 12 h, or 4 h before the biofilm was visible by ethidium bromide staining, there appears to be some sort of debris, presumably organic matter such as proteins, carbohydrates, or other soluble microbial products (Momba et al., 2000), covering portions of the nC_{60} layer. Then, at 24 h, bacteria are clearly visible on the surface. Their somewhat distorted shape is due to the drying and sputter coating. The nC_{60} particles are still visible in the background with the same debris seen at 12 h.

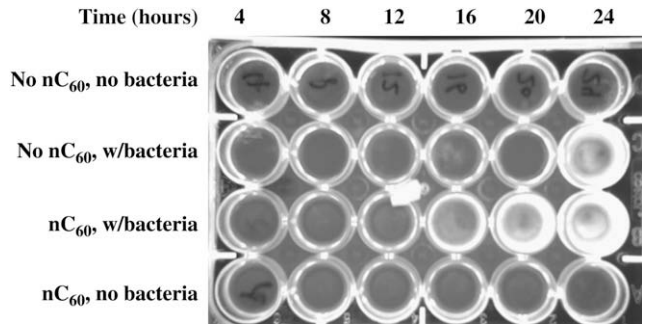

Fig. 1. Biofilm growth in wells shown by UV fluorescence after staining with ethidium bromide.

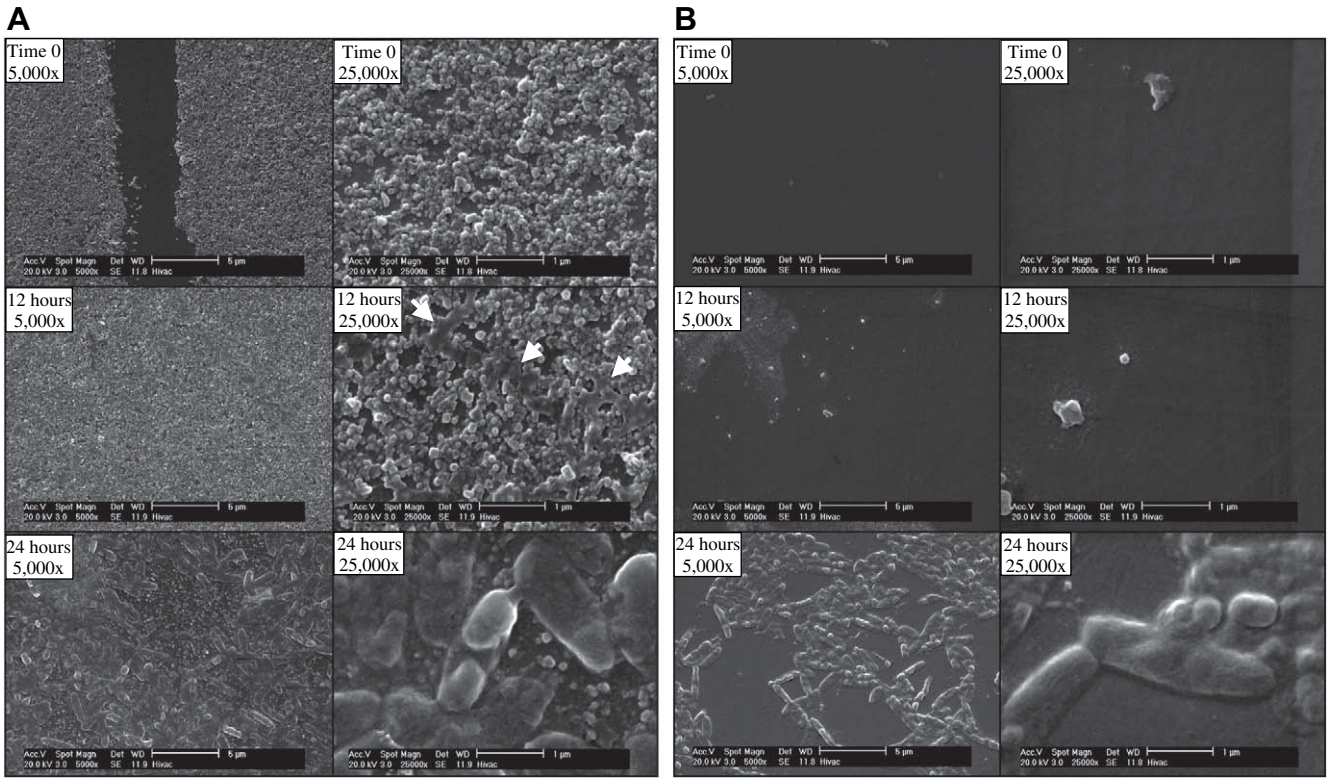

Fig. 2. Time course of biofilm development on (A) nC_{60}-coated polystyrene plates and (B) uncoated polystyrene plates as a control. Micrographs in the left column are taken at 5000× magnification, while those in the right column are taken at 25,000×. The arrows indicate the debris which coats the nC_{60} surface.

A separate experiment was performed to assess the viability of the bacteria that start the biofilm colonization process and determine whether the first cells to colonize the nC_{60}-coated surface die rapidly and form a protective cover for subsequent biofilm formation. Using a live/dead cell staining technique, it is possible to monitor the state of the bacteria as they adhere to the surface. The bacteria were incubated with the nC_{60}-covered surface for various times, stained with CTC and DAPI, and then analyzed by fluorescence microscopy. The number of live versus total bacteria was counted, and these numbers are shown in parentheses in their respective bars of Fig. 3. At 30 min after the introduction of the bacteria, the nC_{60} samples had more bacteria on the surface (although this is not statistically significant) and significantly more of those bacteria were actively respiring. The nC_{60} coating not only allowed more bacteria to colonize, but it also apparently preserved the viability of those bacteria.

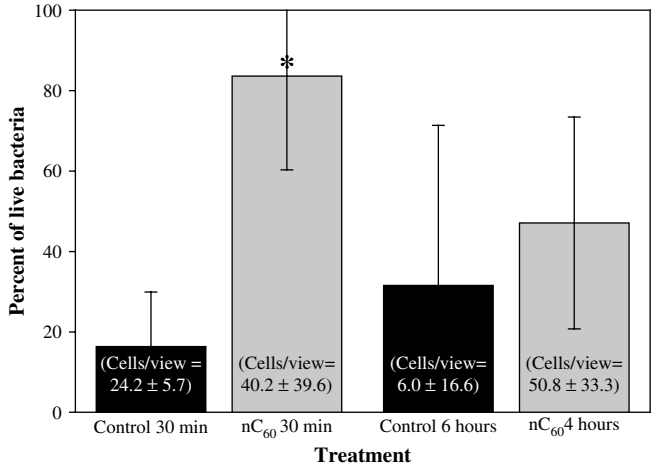

Fig. 3. Percentage of live bacteria involved in biofilm formation in control (no coating) or nC_{60}-coated plastic wells at various times. Numbers in the bars indicate the average number of bacteria per field of view counted. * denotes that results are significantly different from control at the 95% confidence level.

Surprisingly, the number of cells per field of view did not increase significantly between 30 min and 4 or 6 h. This stasis in cell number can be attributed to the reversible adhesion of bacteria that occurs at the beginning of biofilm formation (Stoodley et al., 2002). It was already shown in Fig. 1 that macroscopic biofilm growth does not form until almost 24 h after inoculation, so 6 h is too early to observe irreversible adhesion of bacterial cells. After 4 or 6 h, the percentage of live bacteria in both the control and nC_{60} samples is almost equal, indicating a decrease in viability of the cells in the nC_{60}-coated wells. Whether this was due to residual antibacterial activity of nC_{60} could not be determined.

The failure of the nC_{60} coating to prevent biofilm formation cannot be explained based on what is currently known about the nC_{60} antimicrobial properties. It could be that nC_{60} loses its antibacterial activity when adhered to a surface. It was shown previously that larger nC_{60} particles had lower antimicrobial activity than smaller particles and even lost antimicrobial activity when precipitated by salts (Lyon et al., 2006). Apparently, by coating nC_{60} onto the surface, the particles do not interact as much with the bacteria as they would when suspended. Furthermore, the coated nC_{60} particles do not resuspend in the medium, despite their originally being suspended in water. Once it lost activity, the nC_{60} coating simply provides increased surface area and a rougher and more hydrophobic surface than the control, which would allow the bacteria to attach more easily and grow more plentifully (Pasmore et al., 2001; Harris et al., 2007).

Several factors could account for the apparent lack of antibacterial activity of the nC_{60} coating. nC_{60} has been shown to lose its antibacterial activity in the presence of activated carbon and humic

acids (Dong Li, Rice University, MS thesis), with the hypothesis that bioavailability of the nC_{60} particles decreases as they are sorbed to carbon, trapped in humic colloids, or covered by humic substances. In this case, the nC_{60} particles are still visible (Fig. 2) and thus appear bioavailable, but they may require direct contact to exert their antibacterial effects. The heating process used to create the coating may also have decreased antibacterial activity. However, the most probable explanation for the loss of antibacterial activity is that the debris depicted in Fig. 2A (12 h) (possibly consisting of soluble microbial products and exopolysaccharides) provided a protective shield. The debris would have to deposit quickly, occluding the toxic sites as the cells were reversibly adhering to the surface half an hour after inoculation (Fig. 3). In water distribution systems, organic matter accumulation on metal surfaces allows bacteria to subsequently attach and form biofilms (Momba et al., 2000). This accumulation of organic matter on the surface has been referred to as a conditioning film (CF); the composition of the CF can greatly influence the formation of the biofilm (Marshall, 1994) and pose a significant challenge to the efficacy of any antifouling particle coating.

In summary, current antibiofouling methods fall short in longevity and effectiveness against existing biofilms. The most effective antibiofoulant, tributyl tin, wreaks havoc on marine ecosystems; the industry is in dire need of new and improved antibiofoulants. To this end, the current research effort was undertaken using the potent antimicrobial agent nC_{60}. However, nC_{60} was not an effective antibiofouling agent as applied here. This unexpected result illustrates that some nanomaterials with potent antibacterial properties in suspension may lose their antibiotic activity upon attachment to a surface. Paradoxically, nC_{60} may serve as a biofilm promoter in applications that require biofilm formation, such as certain microbial fuel cells (Dulon et al., 2007). Aerobic biofilms may also be desirable to prevent corrosion of steel surfaces, by reducing the amount of oxygen that reaches the steel plate (Jayaraman et al., 1997). Future research should investigate the reason for the loss of antimicrobial activity once nC_{60} is coated onto the surface. Understanding the mechanisms for this loss of activity might enhance the development of approaches to respond to C_{60} releases and mitigate potential impacts to microbial activities that are important to the health of ecosystems. Currently, the application of nC_{60} as an antifouling coating is not recommended.

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